surface of the antennal lobes through a small window cut in the head cuticle; these experiments were done without the experimenter knowing whether the drop contained PCT or saline. In the second method (group 2, Table 1), 0.1 nl saline or picrotoxin (100 μM–1 mM in saline) was injected directly into the antennal lobes through a small window in the head just above the base of each antenna using a Picospritzer (General Valve)[26]. Injections gave the same results as topical applications, although PE response rates were reduced, as commonly observed after extensive surgery. After a time t_1 (10, 45, 60 or 90 min) for recovery, animals were trained by using the following protocol[11,14]: 6 paired presentations of odorant (4-s pulse into a vented air stream) and sucrose (0.4 μl of 1.25 M solution for group 1, 2 μl of 2 M solution for group 2, presented to the antenna and the proboscis 3 s after odorant pulse onset), every 2 min (group 1) or 30 s (group 2). Animals showing a PE response in each trial were selected to receive 2 or 3 extinction (odour only) trials (one with each of the 2 or 3 test odours; see below) 90 min (group 1) or 60 min (group 2) after conditioning. The odorants used for conditioning were 1-hexanol or 1-octanol. Groups were counterbalanced to contain roughly equal numbers of bees trained with either alcohol. The odours used for testing (1-octanol, 1-hexanol, geraniol) were presented to each animal in a randomized order. Generalization between the alcohols and geraniol is typically low[16]. We used the percentage of subjects that responded to an extinction test as the response measure. Results were compared with χ^2 statistics because behavioural data were categorical (PE or no PE). Statistical values are one-tailed because generalization responses were not expected to exceed the response levels to conditioned stimuli.

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Pri n (PrP^{Sc})-sp cific pitop defined by a monoclonal antibody

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Prions are infectious particles causing transmissible spongiform encephalopathies (TSEs). They consist, at least in part, of a isoform (PrP^{Sc}) of the ubiquitous cellular prion protein (PrP^{C}). Conformational differences between PrP^{C} and PrP^{Sc} are eviden from increased β-sheet content and protease resistance in PrP^{S} (refs 1–3). Here we describe a monoclonal antibody, 15B3, tha can discriminate between the normal and disease-specific form of PrP. Such an antibody has been long sought as it should b invaluable for characterizing the infectious particle as well as fo diagnosis of TSEs such as bovine spongiform encephalopathy (BSE) or Creutzfeldt–Jakob disease (CJD) in humans. 15B specifically precipitates bovine, murine or human PrP^{Sc}, but no PrP^{C}, suggesting that it recognizes an epitope common to prion from different species. Using immobilized synthetic peptides, w mapped three polypeptide segments in PrP as the 15B3 epitope. I the NMR structure of recombinant mouse PrP, segments 2 and 3 of the 15B3 epitope are near neighbours in space, and segment 1 i located in a different part of the molecule. We discuss model for the PrP^{Sc}-specific epitope that ensure close spatial proximity o all three 15B3 segments, either by intermolecular contacts i oligomeric forms of the prion protein or by intramolecula rearrangement.

PrP-null mice were immunized with full-length recombinan bovine PrP. After fusion of spleen cells with myeloma cells, w selected ~50 hybridoma cells that produced monoclonal antibodie recognizing either native bovine PrP^{Sc} (PrP^{BSE}) immobilized o nitrocellulose or recombinant bovine PrP (rbPrP) in an enzyme linked immunosorbent assay (ELISA). One of these antibodie (15B3) was selected for binding to protease-digested BSE brai homogenates; a second (6H4) efficiently recognized recombinan PrP. On western blots, 6H4 recognized rbPrP, as well as bovine human, mouse and sheep PrP^{C}, whereas 15B3 did not react with an form of PrP (results not shown). To determine the reactivity of thes antibodies with native PrP^{C} and PrP^{Sc}, we immunoprecipitated Pr from brain homogenates of normal and BSE-infected cattle. The precipitated proteins were then analysed on western blots using a rabbit polyclonal antiserum to rbPrP (Fig. 1). The 6H4 antibody precipitated PrP from BSE as well as from normal brain homogenates; 15B3 precipitated only PrP from brain homogenates of BSE-diagnosed cattle (Fig. 1a). Upon proteinase K treatment, normal PrP is completely digested, whereas the 33K–35K form of PrP^{Sc} is shortened to 27K–30K (PrP 27–30), probably as a result of

degradation of the amino-terminal segment of residues 23–90, analogous to hamster PrP^{Sc} (ref. 3). Digestions of brain homogenates or immunoprecipitates with proteinase K are shown in Fig. 1b. Proteinase K digestion of BSE homogenates or the immunoprecipitate with 15B3 yields the PrP^{BSE}-specific band of 27K–30K (Fig. 1b). Not all of the immunoprecipitated PrP was protease-resistant, suggesting that 15B3 recognizes multiple forms of disease-specific PrP with different sensitivities to proteinase K. Apparently, PrP with properties characteristic for PrP^{Sc} but without protease resistance occurs as an intermediate in the generation of fully proteinase-resistant PrP^{Sc} (ref. 4). No PrP 27–30 was found in normal homogenates or immunoprecipitates with protein A only (Fig. 1b) or 6H4 (not shown). 15B3 therefore seems to be a PrP^{BSE}-specific antibody, even though we immunized with recombinant bovine PrP. Injection of rbPrP into Tg20 mice overexpressing mouse PrP^{C} has not produced disease for 430 days, whereas two out of four mice injected with a homogenate from the medulla of a BSE-affected cow have come down with TSE at 388 and 426 days (A.R., C.K. and B.O., unpublished results). In addition, it has been reported that recombinant PrP is not infectious[6]. Recombinant PrP is also not protease-resistant, which is a hallmark of PrP^{Sc} (C.K., unpublished observation)[7,8]. The model of Lansbury and Caughey[9], which postulates that the two isoforms of PrP are in a dynamic equilibrium, provides a possible explanation for these findings. By immunizing with large amounts of normal PrP, a small portion of the protein might, according to this hypothesis, have been in the scrapie-specific conformation when triggering the immune response. Alternatively, recombinant PrP molecules might transiently associate (see below and Fig. 3b), and thereby form the prion specific epitope when acting as an immunogen.

We further analysed the species specificity of 15B3 using mouse scrapie-infected brain homogenates (Fig. 1c) and brain homogenates from CJD type-1 patients (Fig. 1d)[10]. For comparison, the mouse brain homogenates of PrP-null as well as normal and scrapie-infected wild-type mice, and the immunoprecipitates corresponding to twice the amount of the homogenates are shown (Fig. 1c). Mouse PrP^{Sc} could be efficiently precipitated by 15B3, as indicated by the presence of PrP 27–30 after the digestion with proteinase K (Fig. 1c, lane a). When brain homogenate was treated with proteinase K before the precipitation, 15B3 was also able to precipitate PrP 27–30 (Fig. 1c, lane b), indicating that the N-terminal segment 23–90 is not critical for binding of 15B3 to PrP^{Sc}, even though precipitation of intact PrP^{Sc} appeared to be more efficient than that of PrP 27–30. Proteinase K digestion causes the formation of large aggregates (scrapie-associated fibrils) which may mask the 15B3 epitope. Surprisingly, 15B3 also specifically recognized PrP^{CJD} from sporadic CJD cases but not human PrP^{C} (Fig. 1d) even though the amino-acid sequence in the regions of the 15B3 epitope is not fully conserved (see below, and Fig. 2b).

The epitopes recognized by the two antibodies were determined by using a gridded array of synthetic peptides consisting of 104 13-residue peptides sequentially shifted in steps of two amino acids and covering the whole mature bovine PrP sequence. A single linear epitope (DYEDRYYRE; corresponding to positions 144–152 of human $PrP^{[11]}$) was mapped for 6H4, whereas three distinct peptide sequences were found to form the 15B3 epitope (amino acids

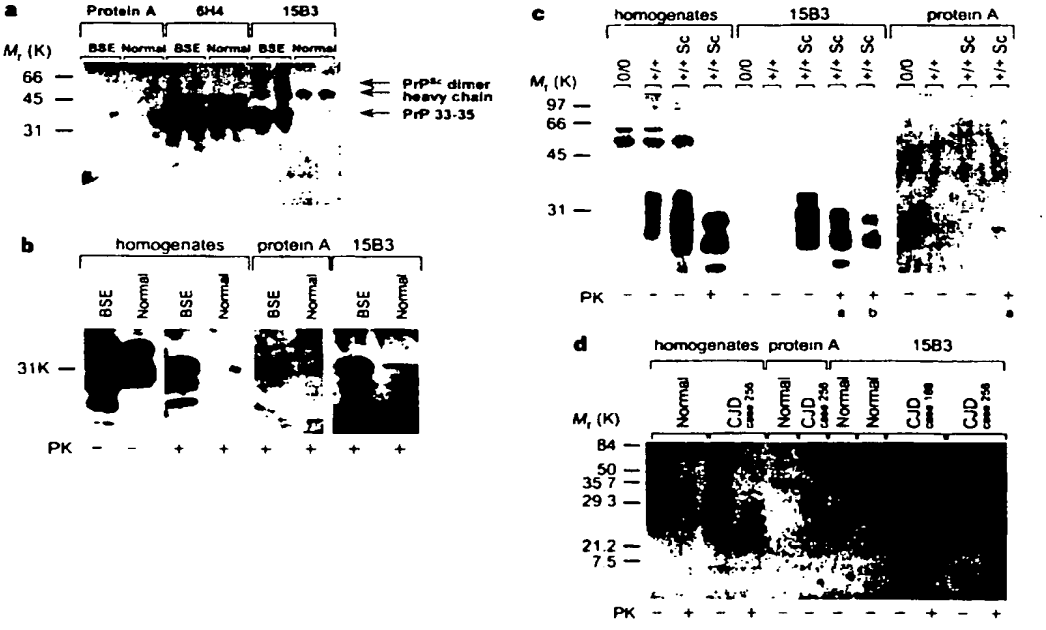

Figure 1 Immunoprecipitation of bovine, mouse and human PrP with monoclonal antibodies 15B3 and 6H4 **a**, The supernatant of a centrifuged homogenate from the medulla of two different BSE-diagnosed or two normal animals was incubated with antibodies 6H4 or 15B3. Antibodies were precipitated with protein A- (15B3) or protein G-agarose (6H4). As a control, protein A only was incubated without antibodies. Precipitates were analysed on a western blot for the presence of PrP using a polyclonal rabbit antiserum to bovine PrP and goat-anti-rabbit Ig coupled to alkaline phosphatase. Signals were developed with chemiluminescence substrates. Crossreaction of the secondary antibody with immunoprecipitated mouse immunoglobulins leads to the prominent band at about 50K. Note the 60K band characteristic for PrP^{Sc} in the 15B3 but not in the 6H4 immunoprecipitations[5]. **b**, Proteinase K digestion of PrP^{BSE} immunoprecipitated with mA 15B3. Undigested and digested bovine brain homogenates were compared to proteinase K digested immunoprecipitates with protein A-agarose only or with 15B3. The sharp band at 31K represents a crossreactivity of the secondary antibody with proteinase K. The same immunoprecipitates and method of analysis were used as in **a**. **c**, Immunoprecipitation of mouse PrP^{Sc} with mAb 15B3. Homogenates from PrP-null mice (0/0) or wild-type mice (normal (+/+) or scrapie-infected (+/+Sc)) were immunoprecipitated with mAB 15B3 or protein A-agarose only and analysed by western blotting as described. Digestion with proteinase K after (a) or before (b) the immunoprecipitation is indicated. Detection of PrP was done as described. **d**, Immunoprecipitation of human PrP^{CJD} with mAb 15B3. Brain homogenates (cerebellum) from normal persons or CJD patients type 1 (ref. 10) were immunoprecipitated and analysed as described for **a**. Two representative examples from a total of 4 normal persons and 4 CJD cases are shown.

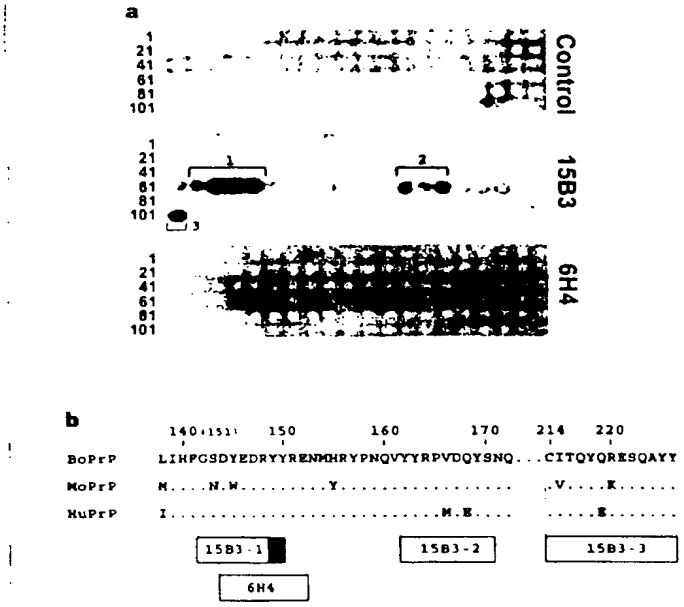

Figure 2 Determination of epitopes for mAbs 15B3 and 6H4. A gridded array of synthetic peptides corresponding to bovine PrP was incubated with 15B3, 6H4 or with secondary antibody only (peroxidase-labelled goat anti-mouse Ig, control). Bound antibody was visualized with chemiluminescence. Each spot corresponds to a 13 amino acid peptide, which is shifted by two amino acids along the bovine PrP sequence relative to the previous peptide. Peptides were covalently attached at the C terminus to a cellulose support. A total of 104 peptides were used to cover the whole bovine PrP sequence including the six octapeptide repeat sequences[17]. **b**, Minimal sequences recognized by 15B3 or 6H4 antibodies in the array of synthetic bovine PrP peptides. The polypeptide segments of the 15B3 epitope are numbered as they occur in the amino acid sequence. The first 15B3 segment extends by two amino acids C-terminally (grey box) if spot number 62, which binds 15B3 only weakly, is excluded. The numbering of the sequence is according to human PrP; the number in brackets indicates the position in the bovine PrP sequences used for the construction of the array of synthetic peptides. Differences with the human and mouse PrP sequences are indicated.

142–148, 162–170, and 214–226; Fig. 2a, b). The relative positic of these partial epitopes in the amino-acid sequence revealed overlap of the 6H4 epitope with the first segment of the 15 epitope (Fig. 2b).

Mapping of the 15B3 epitope onto the NMR structure of the terminal domain of mouse PrP (ref. 12) reveals close proximity the peptide segments 2 and 3, but a much larger spatial separation the segment 1 from either of the other two components (Fig. 3 The peptide segment 1 occupies the N-terminal half of helix 1 pl the two residues preceding it, and it is recognized by 6H4 in PrP^C well as by 15B3 in PrP^{Sc}. This finding would be compatible w either of the two following assumptions: (1) 15B3 recogni: segment 1 of its epitope only in concert with the segments 2 a 3; (2) the polypeptide segment of helix 1 is differently folded in Pr and PrP^{Sc}. Component 2 of the 15B3 epitope is in the lo connecting the second β-strand to the second helix, with t thirds of it in a disordered region in the three-dimensional stru ture, and component 3 is located at the C-terminal end of heli (refs 12, 13). The peptide segments 2 and 3 are located in t proposed binding region for 'protein X' (ref. 14), which is ch acterized by significant alterations of the electrostatic surf potential among different mammalian species[15]: human PrP diff from bovine and mouse PrP in the replacement of the glutami residues 168 and 219 by glutamic acid residues, as well as conservative substitutions at positions 166 and 215. As bovi mouse and human PrP^{Sc} are all precipitated by 15B3 (Fig. 1a– this antibody probably binds to the conserved residues in t region.

A single continuous 15B3 binding site could be formed either aggregation of two or several PrP molecules[16], or by structu rearrangement of a single PrP molecule, or by a combinati thereof. Figure 3b suggests a spatial arrangement of a PrP din that would bring all three segments of the 15B3 epitope into spa proximity, with minimal conformational changes of the individ molecules. It is based on the observation of a structural similar between PrP(121–231) and haemoglobins, which allows a sup position of the helices 1, 2 and 3 of PrP^C onto the helices 1, 6 and 7 the haemoglobin β-subunit, with a root-mean-square distance the polypeptide backbone of 2.4 Å. Superposition of two molecu

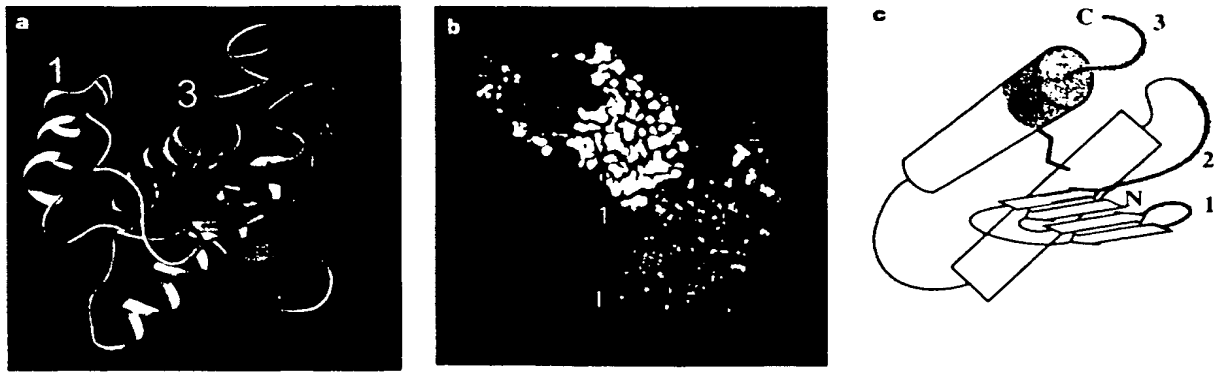

Figure 3 The epitope of the monoclonal antibody 15B3 in the three-dimensional prion protein structure. **a**, Mapping of the 15B3 epitope onto a ribbon drawing of the NMR structure of the C terminal domain PrP(121–231) of mouse PrP^C (refs 12, 26). The three segments of the 15B3 epitope are coloured yellow (1), violet (2) and cyan (3) in the order in which they occur in the amino acid sequence (Fig. 2b). The residual parts of the molecule and the single disulphide bridge are grey. Regular secondary structures are indicated by ribbons for helices and arrows for β-strands. Drawings in **a** and **b** were prepared with MOLMOL[27]. **b**, Surface representation of two PrP(121–231) molecules after superposition onto two β-subunits of the crystal lattice of sickle cell haemoglobin[17] (Protein Data Bank entry 1HBS). The segments of the 15B3 epitope are numbered and coloured as in **a**. The superposition included the backbone atoms of residues 145–154, 179–189, 201–217 of the helices 1, 2 and 3 of PrP(121–231) and of residues 5–14, 106–116, 125–141 of the helices 1, 6 and 7 of haemoglobin S (r.m.s.d. = 2.4 Å). **c**, Hypothetical fold of the prion protein that would bring all three components of the 15B3 epitope into spatial proximity. The two cylinders represent the disulphide-linked helices 2 and 3 in the orientation of **a**. Helix 1 and the β-sheet have been replaced by four β-strands that form a greek key motif. The chain termini are labelled N and C, and the segments of the 15B3 epitope are numbered as in **a**.

of PrP(121–231) onto two adjacent β-chains in the crystal lattice of haemoglobin S[17] (PDB entry 1HBS) brings the peptide segment 1 of one PrP molecule near to the segments 2 and 3 of the other molecule (Fig. 3b). This superposition aligns residue 6 of the β-chains of sickle cell haemoglobin with Trp at position 145 of the mouse prion protein, which is located in the middle of the epitope segment 1 and is fully exposed to solvent. Mutation of Glu 6 to valine is responsible for the formation of haemoglobin aggregates in sickle cell anaemia.

Intramolecular structural rearrangement bringing all three segments of the 15B3 epitope into close spatial proximity might involve the first helix and lead to an extension of the existing β-sheet[13]. In Fig. 3c, the resulting β-structure is assumed to consist of four strands aligned to form a greek-key motif. Other PrP^{Sc} models that would also lead to close approach of the three segments of 15B3 epitope have been described[18].

The identification of an antibody that binds selectively to PrP^{Sc} from various species provides a new means to identify PrP^{Sc} directly without using proteinase K digestion as a criterion. It will be interesting to see whether 15B3 will be able to neutralize infectivity and thus be a potential therapeutic reagent. The low level of PrP^{Sc} in peripheral tissues has made it difficult to use it as a marker for prion diseases[19]. Affinity selection of PrP^{Sc} with 15B3 will allow enrichment of the abnormal isoform of PrP and thus lower the detection limit for PrP^{Sc}, so a prion test for living humans or animals is conceivable. The mapping and three-dimensional modelling of the 15B3 epitopes has provided a view of a prion-disease-specific epitope and may represent a starting point for the production of further diagnostic or therapeutic tools for TSEs. □

Methods

Materials. BSE material was from naturally occurring Swiss cases of BSE, CJD brain material from patients suffering from CJD type 1 (ref. 10), which had been diagnosed using histopathology and immunohistochemistry for PrP. For mouse scrapie material, CD-1 mice were experimentally infected with the RML strain[20].

Preparation of recombinant bovine PrP. The bovine PrP open reading frame was amplified by PCR from genomic DNA using the primers 5′-GGGAATTC-CATATGAAGAAGCGACCAAAACCTG and 5′-CGGGATCCTATTAACTTG-CCCCTCGTTGGTA. The resulting PCR product was cloned into pET11a (Novagen) using the *Nde*I and the *Bam*HI restriction sites. The resulting plasmid (pbPrP3) was transfected into *E. coli* BL21(DE3). Bacteria were grown to $OD_{600} = 0.8$ then induced with 1 mM IPTG and further grown at 30 °C for 3 h. rbPrP corresponding to the mature form of bovine PrP containing six octarepeats[21] was purified from inclusion bodies after solubilization in 8 M urea, 10 mM MOPS/NaOH, pH 7.0 (UM-buffer), on a CM sepharose column (Pharmacia; UM, 0–0.5 M NaCl gradient) and reverse-phase HPLC (Vydac C_4 column, 0.1% trifluoroacetic acid, 0–60% acetonitrile gradient; C.K. and B.O., unpublished results). Resulting fractions contained either oxidized (elution time, 29 min) or reduced PrP (elution time, 34 min). Usually, CM-Sepharose fractions were oxidized with 1 μM $CuSO_4$ for 1 h before purification by reverse-phase HPLC. Purified rbPRP was analysed by mass spectrometry, indicating a protein of the expected mass in which the N-terminal methionine was uncleaved.

Immunization of PrP-null mice. 100 μg recombinant bovine PrP in Freund's complete adjuvant was injected into PrP null mice (mixed background 129/Sv and C57BL/6J[22]) subcutaneously, and 21 and 42 d later with the same amount of antigen in Freund's incomplete adjuvant. Mice were boosted intraperitoneally (day 48) and intravenously (day 49) with recombinant PrP dissolved in PBS. At day 50, mice were decapitated and splenocytes fused to myeloma cells as described[23]. Supernatants of the resulting hybridoma cell lines were screened both by ELISA with recombinant bovine PrP as antigen and by ELIFA[24] using native, protease-digested brain homogenate of BSE-diseased cattle. Positive hybridoma cells were subcloned three times.

Characterization of antibodies. Supernatants of selected hybridomas were used to probe bovine PrP in brain homogenates or recombinant PrP on western blots. To determine the epitopes, antibodies were incubated with a gridded array of peptides comprising 104 polypeptides of 13 amino acids, shifted by two amino acids and covering the entire mature bovine PrP sequence containing six octapeptide repeats[21]. The peptides were covalently attached at their C termini to a cellulose support as individual spots (Jerini Biotools, Berlin). Bound antibody was detected with goat anti-mouse immunoglobulin coupled to horseradish peroxidase and chemiluminescence. Signals were recorded on Hyperfilm ECL (Amersham). For immunoprecipitation, 200 μl 1% brain homogenates (precleared by centrifugation at 13,000*g* for 15 min) were incubated for 2 h at room temperature with 200 μl 0.25 mg ml^{-1} antibody-containing serum-free medium; after incubation with an additional 50 μl protein A- or protein G-coupled agarose (for 15B3 and 6H4, respectively; Boehringer Mannheim) for 2 h at room temperature, agarose beads were centrifuged at 13,000*g* for 3 min and the pellet washed according to the manufacturer. Proteinase K (PK) digestions of immunoprecipitates were done with 20 μg ml^{-1} PK (Sigma) for 30 min at 37 °C. Pellets were then boiled in SDS-sample buffer for analysis on western blots. Immunoprecipitated PrP was detected with polyclonal antibodies raised against bovine recombinant PrP in rabbits (R 26) followed by incubation with a goat anti-rabbit immunoglobulin coupled to peroxidase or a goat anti-rabbit IgG coupled to alkaline phosphatase. Bound enzymatic activity was visualized with chemiluminescent substrates (ECL, Amersham, or CSPD, Tropix, respectively).

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